ENNIATIN SYNTHETASES FROM DIFFERENT FUSARIA EXHIBITING DISTINCT AMINO ACID SPECIFICITIES

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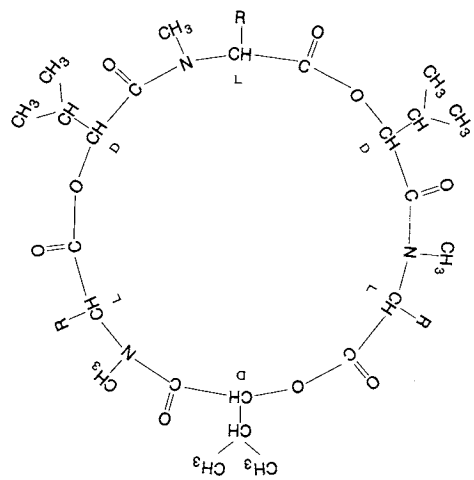
Enniatin synthetases from the cyclodepsipeptide producers *Fusarium lateritium* and *Fusarium sambucinum* were purified to homogeneity and characterized. Like the previously described enniatin synthetase from *Fusarium scirpi* both enzymes consist of a single polypeptide chain and are very similar concerning their Mr (250 kdaltons) and reaction mechanism. Limited proteolytic digests show only slight differences in their patterns in SDS-gels. Interestingly the synthetases differ in their amino acids specificities. The enzyme from the enniatin A producer *F. sambucinum* exhibits a high affinity to the substrate amino acids L-Leu and L-Ile. In contrast the synthetase from the enniatin B producer *F. lateritium* preferably accepts L-Val, the constituent amino acid of enniatin B.

Enniatins are a group of cyclohexadepsipeptides with ionophoretic properties. They are produced by various strains of *Fusarium*[1,2]. As shown in Fig. 1, enniatins consist of either three residues of a branched chain *N*-methyl-L-amino acid and D-2-hydroxyisovaleric acid (D-Hiv) arranged in an alternated fashion. They are synthesized by a multifunctional enzyme consisting of a single polypeptide chain with a molecular mass of about 250 kdaltons, designated enniatin synthetase (ESyn). The precursors of the enniatin molecule (*i.e.* branched chain L-amino acid and D-Hiv) are activated as thioesters. Convalently bound substrate amino acid residues are methylated under consumption of *S*-adenosyl-L-methionine (AdoMet). Then peptide bond formation and cyclization reactions occur[3~6]. From our studies with ESyn from *Fusarium scirpi* we assumed, that the relative amount of enniatin to be synthesized only depends on the pool of the relevant amino acids in the reaction mixture[7] and on the specificity of the enzyme for its substrates. The present paper now provides evidence, that ESyns with different amino acid specificities exist leading to the preferred formation of certain enniatins.

Fig. 1. Structure of enniatins.



Enniatin A: R=–CH(CH$_3$)CH$_2$CH$_3$
Enniatin B: R=–CH(CH$_3$)$_2$
Enniatin C: R=–CH$_2$CH(CH$_3$)$_2$

Materials and Methods

Chemicals and Radioisotopes

S-[Methyl-^{14}C]adenosyl-L-methionine ([methyl-^{14}C]AdoMet), L-[^{14}C]valine, L-[^{14}C]isoleucine, L-[^{14}C]leucine were purchased from Amersham, Braunschweig. Protease from *Staphylococcus aureus* strain V8 (protease V8), α-chymotrypsin,

AdoMet and butyl agarose were from Sigma.

Cultivation of Organisms

F. scirpi strain ETH 1536/9 was cultivated in FDM medium as described[8]. *Fusarium sambucinum* and *Fusarium lateritium* were grown in FCM liquid medium (molasses 3%, corn steep 1%), which was shown to be optimal for these strains concerning enniatin production. Submerged cultures (100 ml medium in 500-ml Erlenmeyer flasks, 120 rpm/minute) were inoculated with 1 ml of a 60 hour-preculture. Mycelium was harvested after about 80 hours.

Enzyme Preparations

ESyn crude extracts from all three enzymes were prepared as described[3,5]. Buffer A (50 mM potassium phosphate buffer, containing 4 mM dithioerythritol) was used throughout the preparations. After the gel filtration on Ultrogel AcA22 ESyn containing fractions were subjected to a hydrophobic interaction chromatography on butyl agarose. ESyn was eluted by a linear gradient of KCl (0~0.3 M) in buffer A. Active fractions (0.18~0.25 M KCl) were collected and concentrated using Centricon 100 ultrafiltration units (Amicon).

Enzyme Assay and Kinetic Measurements

ESyn activity was measured by the formation of radioactively labeled enniatins as described[3]. For kinetic measurements [methyl-^{14}C]AdoMet served as a radiolabel. The initial rate of enniatin formation was measured at varying concentrations of one substrate with fixed saturating concentrations of the others. *Km*- and V_{max}/Km-values were determined from double-reciprocal plots. Radioactive products were separated by TLC on Silica gel plates (Merck) using the solvent system EtOAc - MeOH - H_2O, 100 : 5 : 1 (in volume) and detected by radioscanning or autoradiography.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described[9] using 12%-gels.

Protein Determinations

For protein determinations the BRADFORD procedure was chosen. Bovine serum albumin served as a standard[10].

Affinity Labeling of ESyns

Methylase affinity labeling of ESyns was done as described[11].

Enzyme Linked Immunosorbent Assay (ELISA)

This assay was done as described previously[12]. Polystyrene microtiter plates were coated with ESyn (0.1 ml; 1 μg/ml) in buffer A at 4°C for 12 hours. The well were subsequently incubated with appropriate dilutions of the monoclonal antibody 21.1, with an alkaline phosphatase-conjugated second antibody and with a soln of 4-nitrophenyl phosphate (1 mg/ml) at 37°C. The absorption of formed nitrophenol was measured at 405 nm in a Dynatech multireader.

Results

During our screening of various plant pathogenic strains of *Fusarium* for depsipeptide production we found two positive strains, *F. sambucinum* and *F. lateritium*. HPLC-analysis of EtOAc-extracts of mycelium from these strains revealed, that *F. sambucinum* preferably produces enniatin A (L-Ile as constituent amino acid) and enniatin A1 (2 L-Ile and 1 L-Val), whereas *F. lateritium* and *F. scirpi* preferably produce enniatin B (L-Val as constituent amino acid) and enniatin B1 (2 L-Val and 1 L-Ile). These findings prompted us to isolate and characterize the ESyns from these two strains and compare them with our previously characterized ESyn from *F. scirpi*[3~6]. It was of interest to investigate, whether the different compositions

Table 1. Kinetic constants of ESyns and beauvericin synthetase.

	F. scirpi		*F. sambucinum*		*F. lateritium*		*B. bassiana*	
	Km (μM)	V_{max}/Km*	*Km* (μM)	V_{max}/Km	*Km* (μM)	V_{max}/Km	*Km* (μM)	V_{max}/Km
L-Val	80	1, 81	310	0, 24	53	1, 67	a	
L-Ile	130	0, 35	25	5, 97	77	0, 70	b	
L-Leu	270	0, 19	28	6, 52	770	0, 14	b	
L-Phe	a		a		a		10	8, 06
D-Hiv	5	4, 17	20	9, 31	18	5, 41	1	45, 40
AdoMet	7	34, 40	13	32, 20	3	35, 80	4	17, 50
ATP	350	0, 24	110	0, 65	240	0, 28	100	0, 36

a: No product formation; b: *Km*-values were not determined; * V_{max}/Km (1/second) × 10^4. [Methyl-^{14}C]AdoMet served as radiolabel.

of enniatin-mixtures are the result of different amino acid pools within the fungal cells or may be caused by an altered amino acid specificity of the ESyns.

Purification of Enniatin Synthetases

ESyns from *F. sambucinum* and *F. lateritium* behaved very similar to the enzyme from *F. scirpi* during the purification procedure. This purification included four steps: 1) PEI-precipitation; 2) ammonium sulfate precipitation, (35～45% saturation); 3) gel filtration on Ultrogel AcA 22; 4) hydrophobic chromatography on butyl agarose. As can be seen from Fig. 2-2, after the last purification step the enzymes were obviously homogenous, running in the SDS-PAGE as single bands with an apparent Mr of about 250 kdaltons.

Substrate Specificities of ESyns

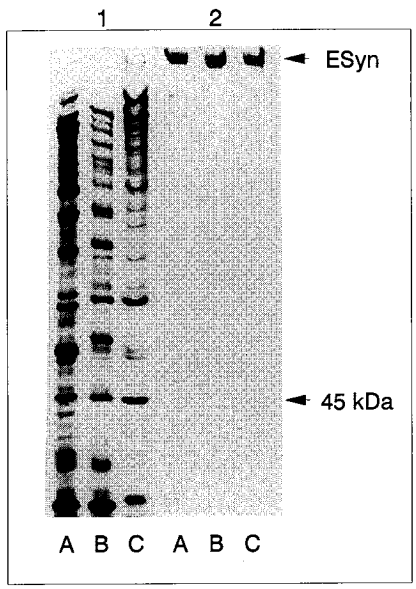
To compare the new synthetases with ESyn from *F. scirpi*[11] concerning their substrate specificities, we measured the *Km*- and V_{max}/Km-values for L-Val, L-Ile, L-Leu, D-Hiv, AdoMet and ATP. As can be seen in Table 1, each of the enzymes has its own kinetic profile. Besides some variations in these kinetic constants for the common substrates AdoMet, D-Hiv and ATP the most striking differences were observed in the affinities of the ESyns to their substrate amino acids: ESyn from *F. lateritium* preferably activates L-Val and thus resembles ESyn from *F. scirpi*, whereas ESyn from *F. sambucinum* exhibits higher affinity to L-Ile and to L-Leu (10- to 30-fold higher V_{max}/Km-values). For comparison the *Km*- and V_{max}/Km-values of beauvericin synthetase from *Beauveria bassiana*[13] are also given. In contrast to the ESyns this enzyme reveals a high specificity for L-Phe as amino acid substrate. In addition it shows 5- to 10-fold higher V_{max}/Km-values for D-Hiv.

Comparison of Proteolytic Patterns of ESyns

The fact, that the three ESyns exhibited different specificities with respect to their substrate amino acids prompted us to compare the proteolytic patterns of the enzymes in SDS-gels. We intended to detect possible differences, that may be responsible for the altered specificities. Therefore we cleaved the enzymes by limited proteolysis using protease V8 and α-chymotrypsin. However the cleavage patterns after SDS-gel electrophoresis were very similar (Fig. 2-1, cleavage with protease V8, lanes A, B, C). As can be seen, there are only slight differences.

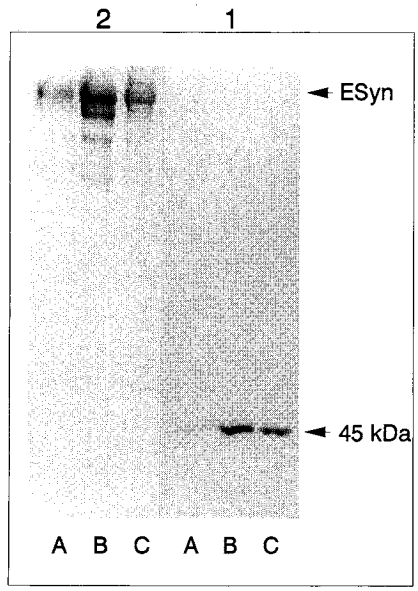
We have recently demonstrated, that the methylase function of ESyn from *F. scirpi*, like other *N*-methyltransferases, can be affinity-labeled in the active site by UV-irradiation in the presence of [methyl-^{14}C]AdoMet. Limited proteolysis using protease V8 yielded a labeled protein fragment[11]. Fig. 3

Fig. 2. SDS-PAGE of different ESyns and their cleavage patterns.



1: ESyns were digested with protease V8 for 90 minutes using 1 μg V8 for 10 μg ESyn in a final soln of 50 μl. Lanes A, B, C: ESyns from *F. lateritium*, *F. sambucinum*, *F. scirpi*, respectively.
12%-SDS-gels were silver stained.
2: ESyns were subjected to SDS-PAGE. Lanes A, B, C; ESyns from *F. lateritium*, *F. sambucinum*, *F. scirpi*, respectively.

Fig. 3. Fluorogram of ESyns and their cleaved fragments after SDS-PAGE.



All 3 ESyns were radiolabeled with [methyl-^{14}C]-AdoMet. The SDS-gel was dried and subjected to fluorography. Lanes: 1A, B, C and 2A, B, C as described in Fig. 2.

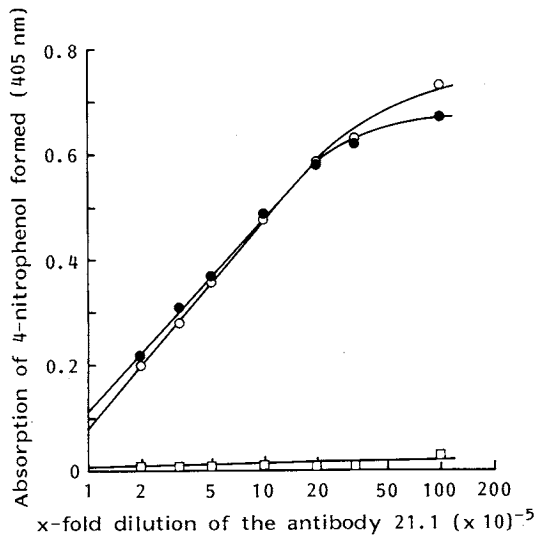
shows the same experiment carried out with ESyns from *F. sambucinum* and *F. lateritium* (ESyn from *F. scirpi* as a control). It can be seen, that in all three cases a labeled protein fragment with a Mr of 45 kdaltons was formed. This fragment was also detected in the silver stain after this proteolysis.

Binding of ESyn-specific Monoclonal Antibodies

We have previously demonstrated, that monoclonal antibodies may be used in structural studies of the multifunctional enzyme ESyn[12]. One of the antibodies (designated 21.1), which specifically inhibited valyl-thioester formation by ESyn from *F. scirpi*, was tested for its binding properties towards the enzymes from *F. sambucinum* and *F. lateritium*. For these studies an ELISA was used. ESyn from *F. scirpi* served as a control. The result is shown

Fig. 4. The binding of the monoclonal antibody 21.1 to 3 ESyns is shown by this ELISA.

○: Reaction of ESyn from *F. lateritium*; ●: Reaction of ESyn from *F. scirpi* (control); □: Reaction of ESyn from *F. sambucinum*.



in Fig. 4. It can be seen, that the enzyme from *F. sambucinum* failed to react with the monoclonal antibody, whereas the enzyme from *F. lateritium*, which preferably activates L-Val, responded like the control.

Discussion

We purified and characterized ESyns from *F. sambucinum* and *F. lateritium*. The enzymes strongly resemble the previously described ESyn from *F. scirpi* with respect to their molecular size and reaction mechanism. Proteolytic patterns of the enzymes were nearly identical, and also the appearance of an affinity-labeled methylase fragment of the same molecular size was observed. In contrast to our previous paper[11] we now found a Mr of 45 kdaltons for the methylase fragment. This may be explained by the fact, that another source of protease V8 was used.

Interestingly the enzymes differ in the specificities towards their substrate amino acids. Obviously the high affinity of the ESyns from *F. scirpi* and *F. lateritium* for L-Val is responsible for the preferred formation of enniatins B and B1 by these strains. The same applies to the amino acid L-Ile, because *F. sambucinum* mostly produces enniatins A and A1.

Another example for a modified ESyn is the recently described beauvericin synthetase from *Beauveria bassiana*[13], which exhibits high affinity to the aromatic amino acid L-Phe. These specificities may be the result of mutational events, altering the amino acid sequence in the amino acid activating sites of the ESyns. The fact, that the valine-site specific monoclonal antibody 21.1 fails to bind ESyn from *F. sambucinum* and beauvericin synthetase[12], supports this assumption.

Work is in progress to isolate and characterize further enzymes of the ESyn family from enniatin producing *Fusaria*.

Acknowledgments

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